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13. SUPPLEMENTARY NOTES

14. ABSTRACT Obesity increases the risk of breast cancer in post-menopausal women. The degree of risk increases proportionally with an increase in adiposity. There is mounting evidence that stromal cells in the tumor microenvironment make pivotal contributions to tumor progression. Adipocytes have been shown to exert their influence on breast cancer cells via two secreted adipocytokines, leptin and adiponectin. Leptin stimulates proliferation and invasiveness of breast cancer cell lines. Conversely, adiponectin is down-regulated in obese individuals, and there is an inverse relationship between adiponectin levels and risk of breast cancer. Therefore, in breast cancer development, it is important to know if there are significant interactions or tumorigenic effects of excess adipose tissue on mammary epithelium. The question of how adipocytes exert their influence on pre-malignant mammary epithelial cells had not been resolved until now. Adiponectin was found to suppress the effects of oncogenic ras or Myc in p53-/- MMECs. These cells slowed their rate of growth in response to adiponectin, and were better able to maintain genomic stability. This lead to reduced transformation potential in a soft agar assay. Conversely, the effects of leptin were very striking. The presence of p53 was able to suppress the oncogenic effects of ras and Myc. However, when leptin was present, oncogene-expressing WT-MMECs behaved just like p53-/- MMECs. These cells had a greatly enhanced rate of growth and became highly aneuploid. Furthermore, p53 was no longer able to function as a tumor suppressor in the presence of leptin, allowing oncogenic transformation.

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Final Report:

Grant Title: Effect of Stromal Adipokines on Breast Cancer Development

PI: Richard A. Woo, Ph.D.

Introduction:

A study in 2002 estimated that 3.2 percent of all new cancers are linked to obesity (1). In the United States, 14 percent of deaths from cancer in men and 20 percent of deaths in women were due to overweight and obesity (2). For breast cancer statistics, the National Cancer Institute reported nearly 210,000 individuals were diagnosed with breast cancer in 2010, and over 40,000 died from the disease (3). Exacerbating the health problem is that overweight and obesity are associated with increased risk of breast cancer in post-menopausal women (4-8). The degree of risk increases proportionally with an increase in adiposity (9). Delineating the impact of obesity on breast cancer is a timely goal as it is predicted 2.3 billion adults will be overweight, and 700 million will be obese within the next 5 years (10). Given the worldwide epidemic of obesity, we sought to understand the molecular mechanism that correlates excess adipose tissue to breast cancer.

In our study, we endeavored to define the interaction between adipocyte/adipokine signaling and breast epithelial cells in order to understanding why obesity is a risk factor for breast cancer. An important and developing area of breast cancer research is the analysis of stromal cells in the tumor microenvironment, and its role in breast cancer etiology (11). It is important to know if there are significant tumorigenic effects of excess adipose tissue beyond the associated increase in estrogen levels. Stromal adipocytes have been shown to exert their influence on breast cancer cells via two secreted adipokines, leptin and adiponectin (12). Leptin, a product of the obese (*ob*) gene and upregulated in obesity, stimulates proliferation and invasiveness of breast cancer cell lines (13). Conversely, adiponectin is down-regulated in obese individuals, and there is an inverse relationship between adiponectin levels and risk of breast cancer (12). Therefore, in breast cancer development, it is important to know if there are significant tumorigenic effects of excess adipose tissue on mammary epithelium. The question of how adipocytes exert their influence on pre-malignant mammary epithelial cells has not

been resolved. It is apparent that concomitant changes at the organismic level (e.g. degree of adiposity and menopausal status) and the tissue microenvironment level (leptin or adiponectin secretion) can vary the effects that adipocytes have on epithelial cells. Conceivably, the epithelial cells may reciprocate and signal back to the adipocytes, or even initiate the signaling.

Hypothesis: It is hypothesized that reciprocal signaling between stromal adipocytes and pre-malignant mammary epithelial cells stimulate epithelial proliferation, which can be a major contributor to the initiating steps required for neoplastic transformation.

Final report in accordance to the statement of work (SOW)

Task 1. Obtain a breeding pair of $p53^{+/-}$ mice, and establish a breeding colony to obtain wildtype and $p53^{-/-}$ mice of female gender. Generate mouse mammary epithelial cells (MMEC) for all subsequent tasks. **(100% completed)**

A single breeding pair of $p53^{+/-}$ mice was obtained (Jax Mice). Following a short quarantine period, the mice were mated, a 20-21 day gestation period ensued, and the first generation of progeny mice was born. After the progeny were weaned (3 weeks after birth), tail tips were snipped for DNA isolation and genotyping. The presence of the p53 and/or nullizygous allele was determined by PCR. When progeny mice were sexually mature (6-8 weeks of age), the breeding was repeated to obtain enough female mice of each genotype for experimentation as well as colony maintenance.

Following genotyping, the desired female mouse of each genotype was dissected. Five mice of each genotype were required to obtain sufficient mammary tissue. In brief, the tissues samples were washed thoroughly with HBSS containing antimicrobial‟s, and finely minced. The tissue suspension was enzymatically digested with collagenase and dispase, followed by centrifugation to remove mesenchymal material and debris. The mouse mammary epithelial cells (MMEC) were cultured on collagen coated plates in serum-free defined growth media (see Figure 1 for details). It is common practice to culture mammalian cells in serum-containing medium. Yet serum's complexity and undefined nature contributes to a situation known as "culture shock", were hyper-

mitogenic signals lead to chromosomal instability, cell cycle checkpoint activation and senescence (14). Since serum-induced chromosomal instability would obscure proposed experiments, serum-free culture conditions similar to what we have used previously (15) were reformulated and refined for MMEC. Wild type ($p53^{+/+}$), $p53^{+/-}$, and $p53^{-/-}$ MMEC were successfully isolated and cultured in serum-free media, and grown using standard primary cell culture techniques (*ibid*).

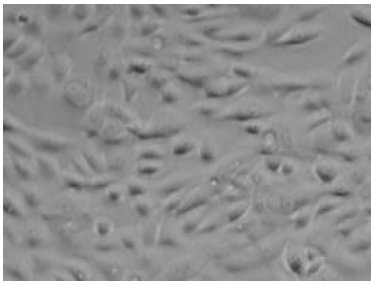


Figure 1. Serum-free culture of mouse mammary epithelial cells (MMEC). MMEC are grown in DMEM:F12 with 26 defined components including EGF, insulin, albumin, transferrin, high density lipoprotein, selenium, glutamine, glutathione and N-acetyl-cysteine. Cells are grown in a humidified, 37^0C incubator at 1% O_2. F broblasts are removed during the first sub-culture with 0.05% trypsin. MMEC remain attached unless trypsinized with 0.25% trypsin + EDTA. By the second passage, the mesenchymal marker, fibronectin was no longer detectable in a protein blot of total cell lysates (data not shown), indicating the culture was epithelial in nature.

The original timeline of 4 months described in the SOW to accomplish this task was underestimated because the first generation of progeny only had a single heterozygous female and a single heterozygous male. In our 8 progeny litter (assuming 50:50 distribution of males and females, as well as Mendelian distribution of the 3 possible genotypes - 1 : 2 : 1 of wildtype : heterozygote : p53 null), ideally, we would have had two heterozygous females and two heterozygous males. With just one of each, colony expansion was slowed by 2-3 months.

Research accomplishment: Establishing a breeding colony of p53 transgenic mice, as well as the generation of $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse mammary epithelial cells (MMEC).

Task 2. To determine the effect of leptin and adiponectin on $p53^{+/+}$ and $p53^{-/-}$ MMEC expressing myc or ras.

(A) Express myc or oncogenic K-rasV12 or both in MMEC. **(100% completed).**

1. The cDNA clone for K-rasV12 and c-myc was obtained from Addgene. We sub-cloned these two genes separately into the retroviral vector, pBMN-GFP (Figure 2; Orbigen). This is a bicistronic vector that expresses green florescent protein (GFP) from an internal ribosome entry site (IRES). The Ψ-promoter controls the constitutive expression of the inserted gene (either K-rasV12 or c-myc). Successful cloning of rasV12 or c-myc into the vector was confirmed by restriction mapping and PCR sequencing (data not shown). The resulting products were designated pBMN-rasV12 and pBMN-Myc.

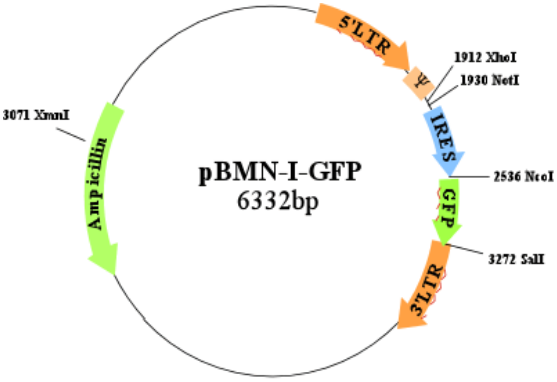
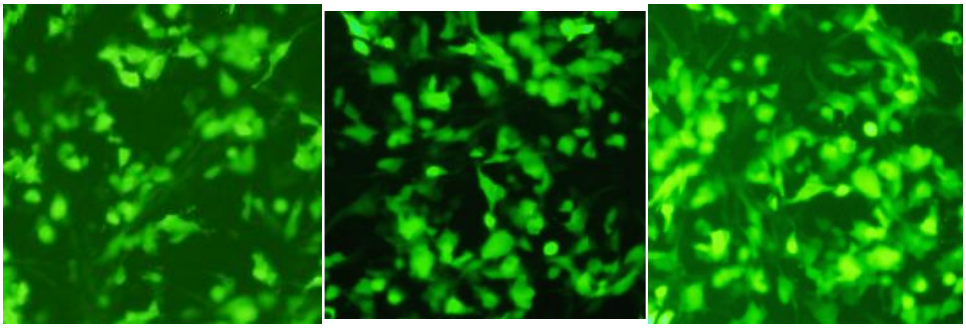


Figure 2. Retroviral vector pBMN-GFP.

Research accomplishment: Construction of recombinant DNA, pBMN-rasV12 and pBMN-Myc.

2. The Phoenix system (Orbigen) produces retrovirus that can efficiently transfer genes to mammalian cells. Retroviral vector constructs, pBMN-rasV12, pBMN-Myc, and parental vector, pBMN-GFP were separately transfected into „Phoenix Eco", ecotropic packaging cells (as well as „Phoenix Ampho" and PT67 amphotropic packaging cell lines – data not shown). Transfected cells were selected in puromycin for 3-4 days, and then analyzed by fluorescence microscopy for the expression of GFP (Figure 3). The resulting cells were designated Eco-GFP, Eco-rasV12 and Eco-myc.



Eco-GFP Eco-rasV12 Eco-myc

Figure 3. Phoenix Eco cells stably transfected with the parental control vector pBMN-GFP, pBMN-rasV12 or pBMN-myc.

Research accomplishment: Generating stable retroviral producer cell lines, Eco-rasV12, Eco-Myc and Eco-GFP.

3. Retrovirus from Eco-GFP, Eco-rasV12 and Eco-Myc cells was used to transduce $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ MMEC generated in **Task 1**.

Retroviral supernatant from Eco-GFP, Eco-rasV12 and Eco-Myc cells was purified by ultra-centrifugation. Retroviral pellets were re-suspended in serum-free MMEC growth media, supplemented with polybrene to enhance retroviral infectivity. MMEC of each genotype ($p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$) was infected with retrovirus coding for either GFP-control, oncogenic rasV12, or c-myc. Infected cells were selected with puromycin, and monitored for virus uptake by fluorescence microscopy, assaying for the GFP marker present in each of the retroviruses. After 2-3 days virtually all of the MMEC showed GFP fluorescence (Figure 4a). The presence of the rasV12 or c-Myc gene was confirmed by PCR (Figure 4b).

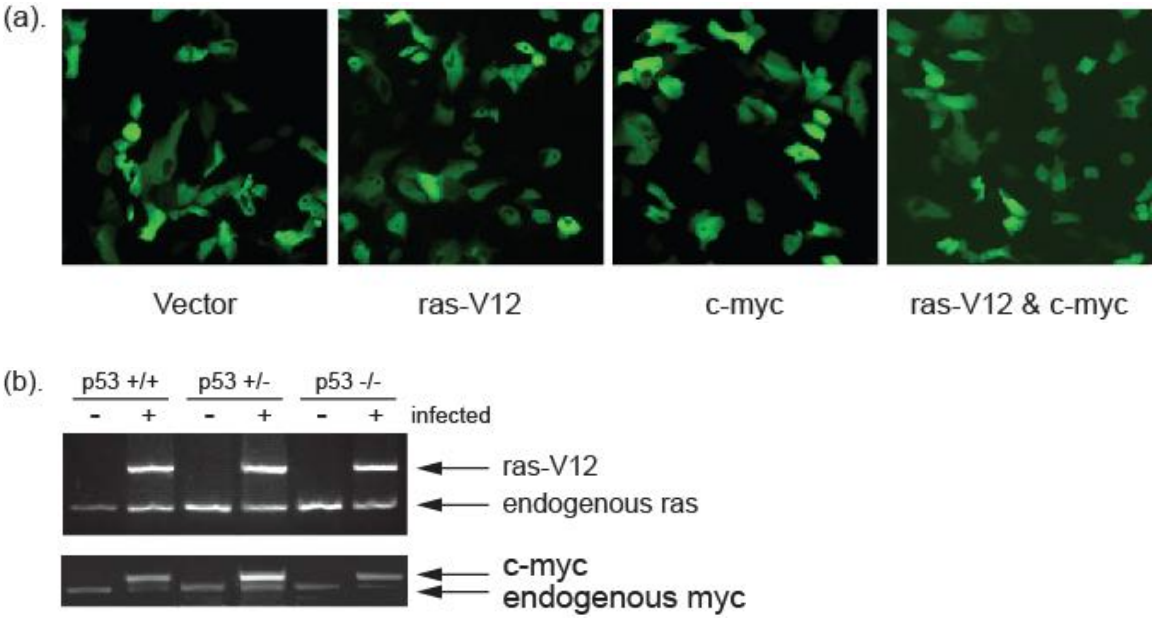


Figure 4. MMEC infected with retrovirus coding for different oncogenes. (a) MMEC were infected with retroviruses containing parental vector, rasV12, c-myc or both rasV12 and myc. Infected cells were then assayed for the expression of GFP by fluorescence microscopy to confirm retroviral infection. (b) Presence of the exogenous K-ras-V12 or c-myc oncogenes were confirmed by PCR. The endogenous murine counterpart was also detected.

Research accomplishment: MMEC were retrovirally transduced with either GFP-control, oncogenic K-rasV12 or c-myc or both rasV12 and c-myc.

(B) To determine the effect of adiponectin or leptin on growth rate, genomic stability and transformation potential of MMEC expressing rasV12 or myc (100% completed).

There is an inverse relationship between adiponectin levels and risk of breast cancer (12). Therefore, we sought to define the effect of adiponectin on mammary epithelial cells. We found that adiponectin had very little effect on the growth rate of non-transduced MMEC, regardless of the genotype (Figure 5 and Table 1). However, the growth rate in oncogene-transduced (rasV12 or myc) MMEC was suppressed by adiponectin (Table 1). In particular, adiponectin was able to nullify the growth advantage conferred by myc in either $p53^{+/+}$ or $p53^{-/-}$ MMEC.

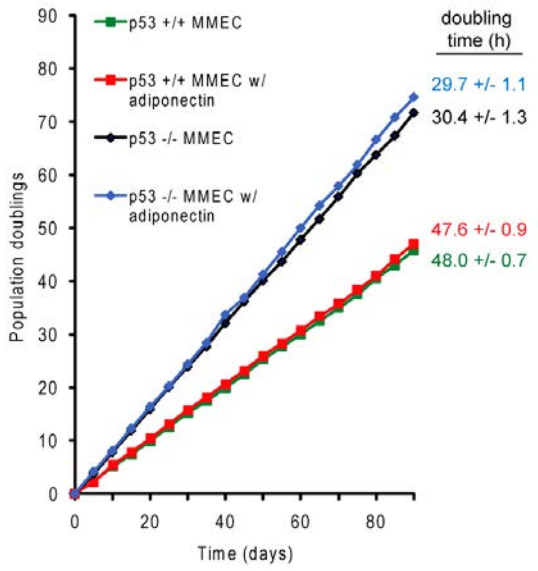


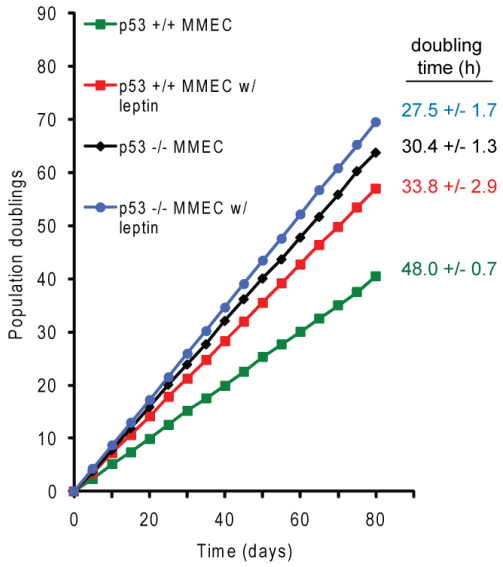
Figure 5. Effect of adiponectin on MMEC growth rate. Cells were either untreated ($p53^{+/+}$ green square; $p53^{-/-}$ black diamond), or cultured in media supplemented with 1ng/ml adiponectin ($p53^{+/+}$ red square; $p53^{-/-}$ blue diamond). Cells were sub-cultured on a regular schedule, and counted using a hemocytometer. Doubling time (+/- SEM) is indicated.

It has been reported that leptin stimulates proliferation in some breast cancer cell lines (13). Similarly, we found that leptin significantly enhances the growth rate of MMEC (Figure 6). The effect of leptin was greatest in cells that retain wild-type p53, decreasing the doubling time by over 14 h. As expected, cells without p53 grow much faster than cells that have p53 (15). Leptin is able to further accelerate the growth of p53 null MMEC, resulting in a ~3 h

Table 1. Effect of adiponectin or leptin on oncogene transduced MMEC

Cells	CTRL	Adiponectin	Leptin
	doubling time (h)		
p53 +/+ MMEC	48.0 ± 0.7	47.6 ± 0.9	33.8 ± 2.9
p53 +/+ MMEC + rasV12	40.1 ± 0.9	44.7 ± 1.7	31.5 ± 2.4
p53 +/+ MMEC + myc	34.2 ± 1.9	43.6 ± 2.1	34.6 ± 2.8
p53 -/- MMEC	30.4 ± 1.3	29.7 ± 1.1	27.5 ± 1.7
p53 -/- MMEC + rasV12	26.4 ± 2.6	28.1 ± 1.3	20.5 ± 3.2
p53 -/- MMEC + myc	21.5 ± 1.4	29.1 ± 2.8	20.1 ± 2.6

Cells transduced with the indicated oncogene were treated as indicated (1ng/ml adiponectin; 1ng/ml leptin). Doubling time (± SEM) is indicated.



decrease in doubling time. Furthermore, leptin hyper-stimulates the growth of MMEC expressing rasV12, regardless of p53 status, but does not enhance the growth stimulating effect of myc (Table 1).

Figure 6. Effect of leptin on MMEC growth rate. Cells were either untreated ($p53^{+/+}$ green square; $p53^{-/-}$ black diamond), or cultured in media supplemented with 1ng/ml leptin ($p53^{+/+}$ red square; $p53^{-/-}$ blue circle). Cells were sub-cultured on a regular schedule, and counted using a hemocytometer. Doubling time (+/- SEM) is indicated.

Aneuploidy and chromosomal instability are hallmarks of virtually all cancers of epithelial origin, including breast cancer. Therefore, we analyzed the karyotypic profiles of the MMEC cultures to assay for gross genomic alterations (Figure 7 and Appendix 1-Supplemental Table S1). Similar to our published data on mouse embryo cell cultures (15), MMEC do not exhibit the „culture shock" phenomenon (14) in our serum-free culture media. Indeed, even the absence of p53, MMEC are predominantly diploid with the normal 40 chromosomal complement (compare Fig. 7a and Fig. 7m). In general, MMEC with p53 are diploid and chromosomally stable, even in the presence of one or two activated oncogenes (Fig. 7a to 7d). Conversely, for $p53^{-/-}$ MMEC, a single „hit" with either rasV12 or myc was more effective in inducing chromosomal instability (Fig. 7m to 7o). With two „hits" from both rasV12 and myc, the majority of the $p53^{-/-}$ MMEC became aneuploid (Fig. 7p). We found that adiponectin was unable to attenuate the genome destabilizing effect of myc, and only mildly reduced the degree of aneuploidy in rasV12-expressing cells.

Leptin, on the other hand was potent in its effect on the genome. Leptin nullified the genome protecting effect of p53 in rasV12-expressing $p53^{+/+}$ MMEC; these cells showed chromosomal instability much like $p53^{-/-}$ MMEC with rasV12 (compare Fig. 7j and 7n). In the absence of p53, leptin on its own leads to aneuploidy (compare Fig. 7m to 7u). In addition, leptin cooperates with rasV12 to significantly enhance the degree of chromosomal instability regardless of p53 status (compare Fig. 7b, 7i, and 7j, or Fig. 7n,

7u and 7v). Interesting that leptin does not add significantly to the genome-destabilizing effect of myc when p53 is not present (compare Fig. 7o to 7w). The cooperative effects of rasV12 and leptin (similar to the cooperative effects of rasV12 and myc), and the non-cooperative effect of myc and leptin (in the absence of p53) suggests that leptin may signal through both the p53 and myc pathways to affect chromosomal instability.

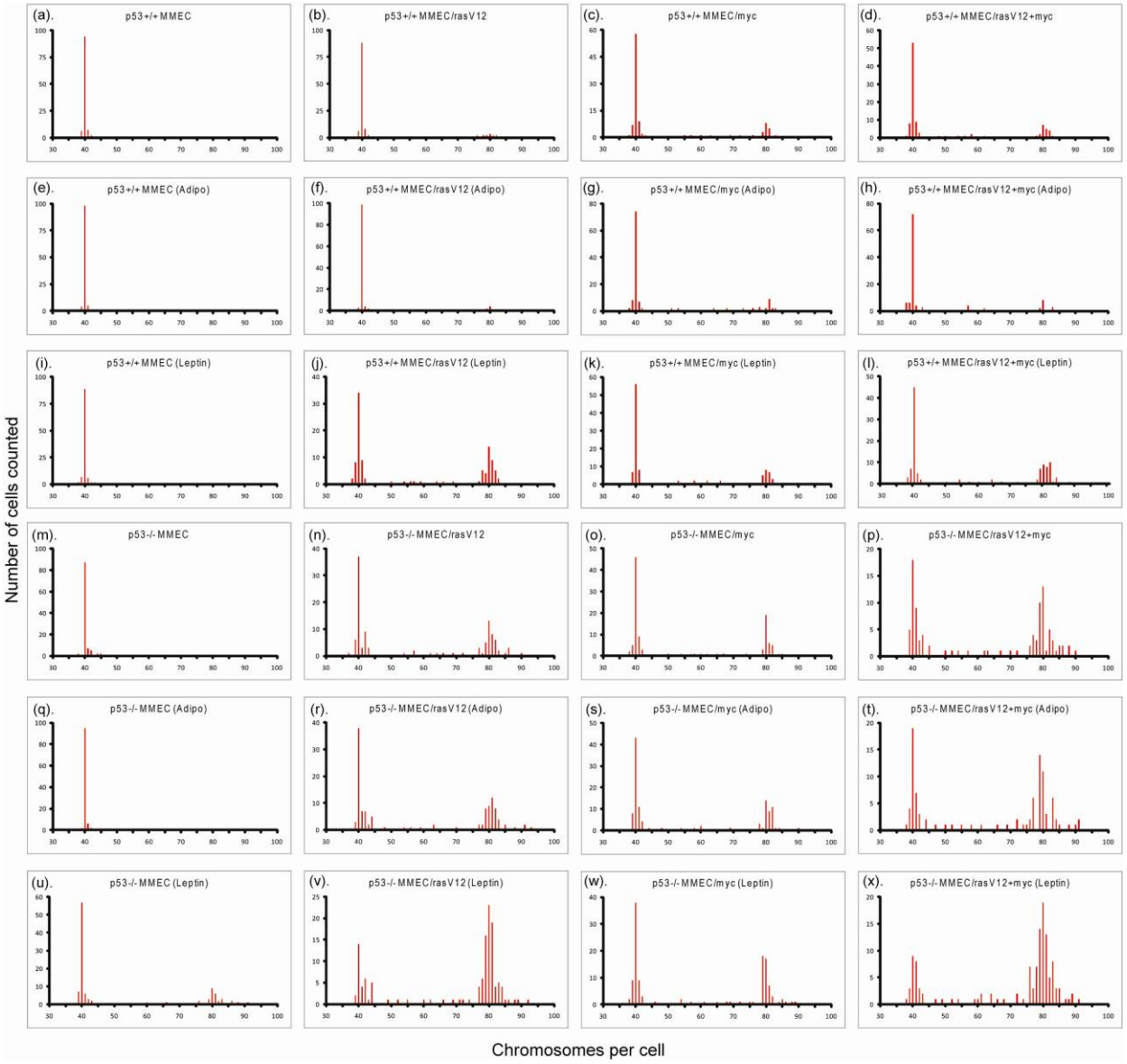


Figure 7. Leptin promotes chromosomal instability in cooperation with activated oncogenes. $p53^{+/+}$ MMEC (panels a to l) or $p53^{-/-}$ MMEC (panels m to x) were cultured in serum-free medium supplemented with 1ng/ml adiponectin or 1ng/ml leptin (as indicated in each panel). The cells were infected with retrovirus expressing vector control (panels a, e, l, m, q, u), rasV12 (panels b, f, j, n, r, v), myc (panels c, g, k, o, s, w), or both rasV12 and myc (panels d, h, l, p, t, x). Two population doublings after retroviral infection (40-100 h, depending on the cell lineage), the cells were treated with nocodazole to induce metaphase arrest for metaphase spreads. Between 100 and 130 metaphase spreads were counted in each case.

A hallmark of in vitro transformation is the ability to grow independent of anchorage. Therefore, we sought to determine the effect of leptin or adiponectin on MMEC colony formation. To that end, we assayed for in vitro transformation as determined by the ability to grow in semi-solid media (Table 2). We found that adiponectin could significantly suppress colony formation of $p53^{+/+}$ MMEC expressing rasV12 and myc, but could not suppress colony formation if p53 was not there, implying that adiponectin signals through the p53 tumor suppressor pathway. Equally intriguing was the observation that leptin stimulates colony formation in cooperation with rasV12, but not myc. This effect was observed whether p53 was present or not, but was more pronounced in the absence of p53. This suggests that leptin is capable of overcoming the tumor suppressor function of p53.

Table 2. Effect of leptin and adiponectin on anchorage independent growth

Cells	Exogenous genes	Colonies /plate		
		Control	Leptin	Adiponectin
p53 +/+ MMEC	control	0	0	0
	rasV12	0	525 ± 170	0
	myc	0	0	0
	rasV12 + myc	395 ± 90	1440 ± 345	30 ± 10
p53 -/- MMEC	control	0	0	0
	rasV12	655 ± 175	5355 ± 935	745 ± 240
	myc	2420 ± 650	2950 ± 455	2425 ± 425
	rasV12 + myc	4125 ± 710	7435 ± 1045	3975 ± 590

10^5 cells were seeded in 3ml of 0.35% low melting point agarose plus growth media, then cast onto 60-mm plates with 3ml of 0.5% agarose in growth media as an underlay. Fresh top agarose in growth media was added regularly as needed. The growth media was supplemented with leptin or adiponectin (1ng/ml) for the indicated cultures. The number of colonies per 60-mm plate was determined by counting the number of colonies in 9 circles with a 10-mm diameter, then calculating the total number of colonies over the entire surface area of the plate. The data represents the mean average from five plates ± standard deviation.

Research accomplishments: Adiponectin is anti-proliferative, suppressing the growth promoted by either rasV12 or myc. It can mildly reduced chromosomal instability promoted by rasV12, and effectively blocks oncogenic transformation in a p53-dependent manner.

Leptin is mitogenic, greatly enhancing the growth rate of mammary epithelial cells, in multiple genetic backgrounds. It nullifies the genome protecting effect of p53, leading to gross chromosomal instability. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc.

Task 3. To test the effect co-cultured adipocytes (3T3-L1) have on proliferation, genomic stability and in vitro transformation of $p53^{+/+}$ and $p53^{-/-}$ MMEC expressing myc or rasV12. **(100% completed)**

3T3-L1 cells are pre-adipocytes that can differentiate into adipocytes upon exposure to dexamethasone, methyl-isobutyl-xanthine, and insulin (16). Undifferentiated 3T3-L1 cells expressed a higher level adiponectin than differentiated 3T3-L1 cells (Figure 8).

Conversely, differentiated 3T3-L1 cells can be stimulated to produce leptin if the growth media is supplemented with insulin (17), confirmed here. These preliminary results should allow us to analyze the different effects of co-cultured undifferentiated 3T3-L1 cells versus differentiated 3T3-L1 cells. Based on previous results adding purified adiponectin or purified leptin (Task 2), there is significant potential in co-culturing adiponectin-expressing 3T3-L1 cells and leptin-expressing 3T3-L1 cells with the MMEC.

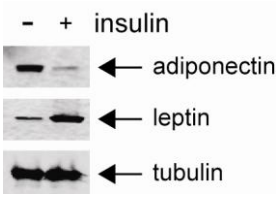


Figure 8. Expression of adiponectin and leptin in 3T3-L1 cells. Cells analyzed were undifferentiated 3T3-L1 cells (-) or differentiated 3T3-L1 cells cultured with 10 μg/mL of insulin (+). Total cellular lysate from harvested cells were immunoblotted for adiponectin or leptin. Uniform gel loading was confirmed by immunoblotting for tubulin.

For co-culture experiments, MMEC were cultured in 6-well plates. 3T3-L1 cells were co-cultured in a transwell insert (Corning) above the MMEC. The results of the co-culture experiments were in general agreement with the studies using purified adiponectin and leptin (Table 3). Co-cultured undifferentiated 3T3-L1 cells (adiponectin-expressing cells) had very little effect on the growth rate of non-transduced MMEC, regardless of the genotype. However, the growth rate of myc expressing p53$^{-/-}$ MMECs was suppressed by co-cultured undifferentiated 3T3-L1 cells. The most significant effect of undifferentiated 3T3-L1 cells was on rasV12 or myc expressing p53 $^{+/+}$ MMEC. Here the growth rate was decreased by 50- 100%. Perhaps the drastic decrease in cell growth was due to cell death observed in the p53 $^{+/+}$ MMEC culture plates (data not shown), but not the p53 $^{-/-}$ MMEC. If so, this would suggest adiponectin induced a p53-dependent apoptotic response, perhaps through p19^{ARF} (18). This would not be unexpected since activated oncogenes like rasV12, myc and others sensitize cells to p53-dependent apoptosis (15). By comparison, adiponectin did not produce noticeable cell death in the MMEC cultures. The difference may

Table 3. Effect of co-cultured 3T3-L1 cells on MMEC growth rate

Cells	—	3T3-L1^{ad}	3T3-L1^{lep}
	doubling time (h)		
p53 +/+ MMEC	48.0 ± 0.7	48.7 ± 3.9	40.7 ± 3.2
p53 +/+ MMEC + rasV12	40.1 ± 0.9	64.7 ± 11.7	34.5 ± 4.8
p53 +/+ MMEC + myc	34.2 ± 1.9	73.2 ± 18.4	37.4 ± 5.1
p53 -/- MMEC	30.4 ± 1.3	32.1 ± 3.1	29.4 ± 3.3
p53 -/- MMEC + rasV12	26.4 ± 2.6	26.2 ± 4.3	23.1 ± 2.3
p53 -/- MMEC + myc	21.5 ± 1.4	31.1 ± 4.2	22.0 ± 2.9

MMEC, transduced with the indicated oncogene, were co-cultured with the indicated cells. Data represents MMEC doubling time ± SEM.
[ad] Undifferentiated 3T3-L1 cells producing adiponectin.
[lep] Differentiated 3T3-L1 cells treated with 10 μg/mL to enhance leptin production.

be due to the level of adiponectin produced from the 3T3-L1 cells, or perhaps some other factor, not yet accounted for. Future experiments to test different possibilities include: 1. testing different doses of adiponectin to see if it can induce cell death in MMEC; 2. silence adiponectin in the undifferentiated 3T3-L1 cells by siRNA, to see if another factor in undifferentiated 3T3-L1 cells induced the cell death; 3. confirm the cell death is p53-dependent apoptosis. Next, we co-cultured with differentiated 3T3-L1 cells which express elevated levels of leptin when induced with insulin (17). Leptin-producing differentiated 3T3-L1 cells generally had a mitogenic effect on non-transduced, as well as rasV12-transduced MMECs, but not myc-expressing cells. This was identical to the effect of leptin on the MMEC cultures, confirming that adipocytes can affect the growth of mammary epithelial cells.

To determine the effect of adipocytes on the degree of aneuploidy in co-cultured MMEC, we performed chromosome counts on metaphase spreads (Table 4). The adiponectin-producing adipocytes suppressed the degree of aneuploidy in rasV12 and myc expressing $p53^{+/+}$ MMEC. As discussed earlier, it is hypothesized that adiponectin triggers a p53-dependent apoptotic response in the rasV12 and myc cells. If this is indeed the case, it is reasoned that the low percentage of aneuploidy is due to the aneuploid cells dying by apoptosis. In support of this, adiponectin-producing adipocytes do not suppress aneuploidy in the rasV12 or myc expressing p53 knockout MMEC.

Table 4. Effect of co-cultured adipocytes on chromosomal instability in mammary epithelial cells

Cells	Co-cultured cells	Total cells counted	Percent gross aneuploidy[a]	Cells with indicated chromosome number		
				≤38	39 to 41	≥42
p53 +/+ MMEC	—	109	1.8		107	2
	undiff. 3T3-L1	102	3.9	1	99	3
	diff. 3T3-L1	101	4	2	95	2
p53 +/+ MMEC + rasV12	—	117	12.8		112	15
	undiff. 3T3-L1	108	1.9	1	106	1
	diff. 3T3-L1	120	34.2	2	79	39
p53 +/+ MMEC + myc	—	102	27.5	2	74	26
	undiff. 3T3-L1	109	10.1		98	11
	diff. 3T3-L1	109	25.7	1	81	27
p53 +/+ MMEC + rasV12 & myc	—	101	30.7	1	70	30
	undiff. 3T3-L1	101	11.9	2	89	10
	diff. 3T3-L1	119	37		75	44
p53 -/- MMEC	—	106	11.3	2	94	10
	undiff. 3T3-L1	114	4.4		109	5
	diff. 3T3-L1	112	25.9	1	83	28
p53 -/- MMEC + rasV12	—	110	58.2	1	46	63
	undiff. 3T3-L1	126	53.2	1	59	66
	diff. 3T3-L1	119	78.2		26	93
p53 -/- MMEC + myc	—	109	45	2	60	47
	undiff. 3T3-L1	128	41.4		75	53
	diff. 3T3-L1	117	49.6	2	59	56
p53 -/- MMEC + rasV12 & myc	—	101	68.3		32	69
	undiff. 3T3-L1	115	61.7	1	44	70
	diff. 3T3-L1	121	68.6	2	38	81

[a]We define gross aneuploidy as any cell that has an absolute difference of two or more from the diploid number.

Analysis of the effect of leptin-producing 3T3-L1 cells produced results similar to treatment of MMEC cultures

with leptin. The rasV12-expressing $p53^{+/+}$ MMEC were chromosomally stable (12.8% aneuploid), but become unstable (34.2% aneuploidy) when co-cultured with leptin-producing adipocytes. Similar to leptin treatment, the myc-expressing $p53^{+/+}$ MMEC were not affected by co-cultured leptin-producing adipocytes. Conversely, leptin-producing adipocytes do not compound the effect of myc. Again this is consistent with the prior hypothesis that leptin signals through both the p53 and myc pathways to induce chromosomal instability.

Finally, given the observations that co-cultured leptin-producing adipocytes stimulate proliferation and chromosomal instability in mammary epithelial cultures, the next assay will test adipocyte conditioned media on MMEC colony formation in soft agarose (Table 5). The adiponectin-producing undifferentiated 3T3-L1 cells block in vitro transformation of $p53^{+/+}$ MMEC expressing rasV12 plus myc. The leptin-producing differentiated 3T3-L1 cells cooperates with oncogenic rasV12 to transform MMEC of either genotype.

Table 5. Effect of adipocyte conditioned media on MMEC anchorage independent growth

Cells	Exogenous genes	Colonies /plate		
		control	diff. 3T3-L1	undiff. 3T3-L1
p53 +/+ MMEC	control	0	0	0
	rasV12	0	415 ± 130	0
	myc	0	0	0
	rasV12 + myc	395 ± 90	510 ± 180	0
p53 -/- MMEC	control	0	125 ± 45	0
	rasV12	655 ± 175	2705 ± 435	585 ± 170
	myc	2420 ± 650	2020 ± 375	1925 ± 425
	rasV12 + myc	4125 ± 710	3495 ± 405	3175 ± 680

10^5 cells were seeded in 3ml of 0.35% low melting point agarose plus adipocyte conditioned growth media from the indicated 3T3-L1 culture. The cell suspension was then cast onto 60-mm plates with 3ml of 0.5% agarose in adipocyte conditioned growth media as an underlay. Fresh top agarose also with adipocyte conditioned growth media was added regularly as needed. The number of colonies per plate was determined as described in Table 2.

Research accomplishments: Adiponectin-producing adipocytes elicit an anti-proliferative signal towards mammary epithelial cells, suppressing growth promoted by either rasV12 or myc. It leads to reduced chromosomal instability promoted by rasV12, and effectively blocks oncogenic transformation in a p53-dependent manner.

Leptin-producing adipocytes elicit a mitogenic response, greatly enhancing the growth rate of mammary epithelial cells, in multiple genetic backgrounds. Signaling from co-cultured adipocytes nullifies the genome protecting effect of p53, leading to gross aneuploidy. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc.

Task 4. To investigate if MMEC can „instruct" co-cultured adipocytes to alter the expression or secretion of leptin or adiponectin. **(100% completed)**

We have shown that adipocytes can influence the growth, chromosomal stability and oncogenic transformation of mammary epithelial cells. Next is the question of whether signaling is bidirectional. Do mammary epithelial cells signal to adipocytes, perhaps by priming the stroma to prepare a hospitable tumor micro-environment? Do the signals from mammary epithelial cells differ depending on the stage the cell is at in the multiple steps of tumorigenesis?

To test this, we utilized mouse mammary epithelial cells (MMEC) with one or more activated oncogenes, and/or loss of the p53 tumor suppressor gene to represent the different stages of multi-step tumorigenesis. These cells were co-cultured with 3T3-L1 adipocytes, similar to experiments described above. Samples were collected at regular intervals over a 9 day period (Figure 9a). Normal $p53^{+/+}$ MMEC had no effect on adiponectin production. However, the effect of rasV12 and/or myc expressing $p53^{+/+}$ MMEC on adiponectin-producing adipocytes was striking. The adiponectin levels decreased progressively with time in co-culture. This effect was only observed when the adipocytes were co-cultured with MMEC that have p53. The $p53^{-/-}$ MMEC did not have any effect on adiponectin production (data not shown).

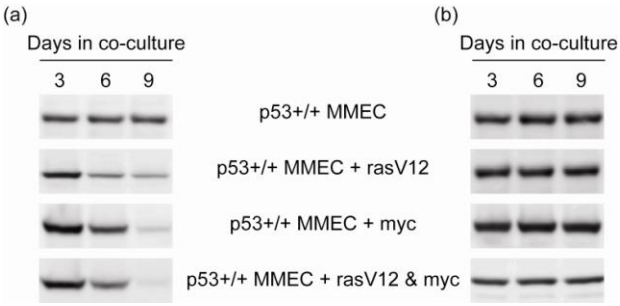


Figure 9. Effect of MMEC on adiponectin and leptin production in co-cultured adipocytes. (a). The indicated MMEC were co-cultured with undifferentiated 3T3-L1 cells for up to 9 days. The adipocytes were harvested at regular intervals, cell extracts were made, and samples were immunoblotted for adiponectin. (b). MMEC were co-cultured with differentiated 3T3-L1 cells in the presence of insulin (10 μg/mL) to permit leptin production. The adipocytes were harvested at regular intervals, cell extracts were made, and samples were immunoblotted for leptin.

Next, MMEC were co-cultured with differentiated 3T3-L1 cells in the presence of insulin to permit leptin production (17). Co-cultured $p53^{+/+}$ MMEC had no effect on leptin production whether rasV12 or myc was expressed or not (Figure 9b). The $p53^{-/-}$ MMEC did not have an effect on leptin production either (data not shown).

Future follow-up experiments will probe the specific signaling pathway that allows MMEC to affect adiponectin production in adipocytes. We would hypothesize that adiponectin induced p53-dependent apoptosis in the MMEC elicits a feedback response on the adipocytes to down regulate adiponectin levels. These experiments are underway, but outside the scope of the current project. Also in the works is the use of primary adipocytes from the mammary fat pad of mice. Using normal primary adipocytes instead of the immortalized 3T3-L1 cell line could be more revealing, particularly if they do not require chemical differentiation or insulin stimulation for leptin production.

Research accomplishments: Signaling between mammary epithelial cells and adipocytes is bidirectional. MMEC expressing activated oncogenes, rasV12 and myc, can signal to co-cultured adipocytes to down regulate adiponectin production. This was dependent on p53.

Key Research Accomplishments

1. Establishing a breeding colony of p53 transgenic mice, as well as the generation of $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ mouse mammary epithelial cells (MMECs).
2. Construction of recombinant DNA, pBMN-rasV12 and pBMN-Myc.
3. Generating stable retroviral producer cell lines, Eco-rasV12, Eco-Myc and Eco-GFP.
4. MMEC were retrovirally transduced with either GFP-control, oncogenic K-rasV12 or c-myc or both rasV12 and c-myc.
5. Adiponectin is anti-proliferative, suppressing the growth promoted by either rasV12 or myc. It can mildly reduced chromosomal instability promoted by rasV12, and effectively blocks oncogenic transformation in a p53-dependent manner.
6. Leptin is mitogenic, greatly enhancing the growth rate of mammary epithelial cells, in multiple genetic backgrounds. It nullifies the genome protecting effect of p53, leading to gross chromosomal instability. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc.
7. Adiponectin-producing adipocytes elicit an anti-proliferative signal towards mammary epithelial cells, suppressing growth promoted by either rasV12 or myc. It leads to reduced chromosomal instability promoted by rasV12, and effectively blocks oncogenic transformation in a p53-dependent manner.
8. Leptin-producing adipocytes elicit a mitogenic response, greatly enhancing the growth rate of mammary epithelial cells, in multiple genetic backgrounds. Signaling from co-cultured adipocytes nullifies the genome protecting effect of p53, leading to gross aneuploidy. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc.
9. Signaling between mammary epithelial cells and adipocytes is bidirectional. MMEC expressing activated oncogenes, rasV12 and myc, can signal to co-cultured adipocytes to down regulate adiponectin production. This was dependent on p53.

Conclusions:

Obesity is a significant risk factor for breast cancer, particularly in post-menopausal women. Until now, the molecular mechanism that governs the role of obesity in breast cancer development and progression was unknown. Here we describe the impact of two adipocytokines, adiponectin and leptin, on mammary epithelial growth, chromosomal instability and oncogenic transformation. Most cancer cells are aneuploid. Oncogenes rasV12 and c-Myc accelerated the growth rate of MMEC leading to chromosomal instability and aneuploidy. These oncogenes also induced in vitro transformation of MMEC. Adiponectin was found to suppress the effects of oncogenic rasV12 or Myc in MMEC. These cells slowed their rate of growth in response to adiponectin, and were better able to maintain chromosomal stability. This lead to reduced transformation potential in a soft agar assay. Since adiponectin is down-regulated in obese individuals, they would not benefit from its protective effects.

Here we define for the first time, how obesity promotes mammary tumorigenesis via the adipokine, leptin. Leptin is mitogenic. Although p53 is able to suppress the oncogenic effects of rasV12 and Myc, when leptin is present, rasV12-expressing $p53^{+/+}$ MMEC behaved just like $p53^{-/-}$ MMEC. These cells have a greatly enhanced rate of growth and become aneuploid. Leptin nullifies the genome protecting effect of p53, leading to gross chromosomal instability. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc. Co-culture of leptin-producing adipocytes also elicited a mitogenic response, greatly enhancing the growth rate of mammary epithelial cells. Signaling from co-cultured adipocytes nullifies the genome protecting effect of p53, leading to gross aneuploidy. This enhances in vitro mammary tumorigenesis promoted by rasV12 and myc.

Co-culture of adiponectin-producing adipocytes triggers an anti-proliferative pathways in mammary epithelial cells, suppressing growth promoted by either rasV12 or myc. It leads to reduced chromosomal instability promoted by rasV12, and effectively blocks oncogenic transformation in a p53-dependent manner. The signaling between mammary epithelial cells and adipocytes is bidirectional. MMEC expressing activated

oncogenes, rasV12 and myc, can signal to co-cultured adipocytes to down regulate adiponectin production. This too was dependent on p53.

The future direction for this project has already been described above. The key to breast cancer development (as well as development of most carcinomas) is chromosomal stability. Therefore, perhaps the most important next step is to decipher how adiponectin and leptin have their effect on aneuploidy. This will include understanding the cell death induced by adiponectin, hypothesized to be p53-dependent apoptosis, and whether there is a feedback loop from oncogene transformed mammary epithelial cells to thwart the protective effects of adiponectin.

Examination of pathways altered in obesity may offer new targets for breast cancer therapy. These therapeutic strategies could include targeting adipokines or their receptors to prevent development of breast cancer or progression of the disease. Leptin (and obesity) may be the 'disease', but adiponectin may be the proverbial 'cure'.

Reportable Outcomes

1) R.A. Woo. Stromal adipokines in breast cancer development. *International Journal of Molecular Medicine: Special Supplement Edition*. October, 2009.
2) Preparation of the Manuscript "Stromal leptin co-operates with oncogenic ras to promote genomic instability and oncogenic transformation in mammary epithelial cells". J.H. Huang, C. Harrison, R.A. Woo. Submitted for publication.
3) Recruitment and training of C. Harrison, Research II Technician.
4) Invited speaker at 14th World Congress on Advances in Oncology, 2009. "Stromal adipokines in breast cancer development"
5) Invited speaker at 12th International Symposium on Molecular Medicine, 2009. "Stromal adipokines in breast cancer development"

Appendix 1

Supplemental Table S1. Effect of adipokines on chromosomal instability in mammary epithelial cells

Cells	Adipokine	Total cells counted	Percent gross aneuploidy[a]	Cells with indicated chromosome number		
				≤38	39 to 41	≥42
p53 +/+ MMEC	—	109	1.8		107	2
	adiponectin	107	0		107	
	leptin	108	5.6	2	102	4
p53 +/+ MMEC + rasV12	—	117	12.8		112	15
	adiponectin	116	8.6		106	10
	leptin	103	50.5	2	51	50
p53 +/+ MMEC + myc	—	102	27.5	2	74	26
	adiponectin	123	27.6	2	89	32
	leptin	108	34.3		71	37
p53 +/+ MMEC + rasV12 & myc	—	101	30.7	1	70	30
	adiponectin	113	27.4	6	82	25
	leptin	112	49.1	3	57	52
p53 -/- MMEC	—	106	11.3	2	94	10
	adiponectin	105	1.9		103	2
	leptin	106	33.9		70	36
p53 -/- MMEC + rasV12	—	110	58.2	1	46	63
	adiponectin	120	60		48	72
	leptin	128	84.4		20	108
p53 -/- MMEC + myc	—	109	45	2	60	47
	adiponectin	114	45.6		62	52
	leptin	123	55	2	56	65
p53 -/- MMEC + rasV12 & myc	—	101	68.3		32	69
	adiponectin	100	70	1	30	69
	leptin	128	83.6	1	20	107

[a]We define gross aneuploidy as any cell that has an absolute difference of two or more from the diploid number.

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